Antibiotic resistance patterns and prevalence of class I, II and III Integrons among clinical isolates of *Klebsiella pneumoniae*

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SUMMARY

Klebsiella pneumoniae is a well-known pathogen and contributes to different types of infection. To investigate the antibiotic resistance profiles and prevalence of class I, II, and III integrons among clinical isolates of *K. pneumoniae*, a total of 142 non-duplicate clinical isolates were collected. Antibiotic susceptibility was assessed using Kirby-Bauer's disk diffusion method and Clinical and Laboratory Standards Institute (CLSI) guidelines. Polymerase chain reaction (PCR) method was used to identify class I, II and III integrons. The isolates were mostly resistant against streptomycin (62 strains, 43.7%) and ceftriaxone (42 strains, 29.6%). Twenty-six (18.3%) isolates were found to be multi-drug resistant (MDR). Class I and II integrons were detected in 65 isolates (45.8%) and 1 (0.7%) isolate, respectively. The findings of this study revealed that the prevalence of streptomycin-resistant isolates is high, and its use must be restricted. Also, our results revealed that class I integrons are widely prevalent in clinical isolates of *K. pneumoniae* and a significant association was observed between resistance against imipenem, ciprofloxacin, gentamicin and streptomycin and the presence of integrons, necessitating appropriate infection control programs.

Keywords: *K. pneumoniae*, integrons, antibiotic resistance.

INTRODUCTION

Klebsiella pneumoniae, especially hypervirulent strains, is an important member of *Enterobacteriaceae* family and contributes to different types of infection, including respiratory tract infections, urinary tract infections (UTI), blood stream (BSI) and wound infections [1]. Hypervirulent *K. pneumoniae* isolates, due to increased secretion of extracellular polysaccharide and toxins such as salmochelin, yersiniabactin and aerobactin, as well as cell-cycle modulating non-ribosomal peptide colibactin and pore-forming proteins, are in association with poor patients' outcomes [2].

The ability of bacteria to capture and disseminate antibiotic resistance determinants by horizontal gene transfer are considered as a successful mechanism in development of multi-drug resistant (MDR) strains, especially among members of *Enterobacteriaceae* family. Plasmids, transposons and integrons are mobile genetic elements that harbor antibiotic resistance genes and can be transferred via horizontal gene transfer mechanism [3].

Integrons are recognized as genetic elements that acquire exogenous genes in the form of mobile gene cassettes and play a pivotal important role in capturing and expressing diverse antimicrobial resistance genes. All integrons consist of three segments including *intI*, known as inte-

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gron-integrase gene encoding a tyrosine recombinase enzyme, promoter site (Pc) and recombination site (*attI*), where integrase inserts new captured resistance gene [4].
Based on differences in sequence of integrase genes five classes of integrons including class I, II, III, IV and V have been reported. However, the last two classes including integrons class IV and V have not been identified in *K. pneumoniae* [3].
Class I integron is the most frequently reported integron among *Enterobacteriaceae*, especially *Klebsiella* spp. Class I integron platforms are composed of two conserved regions called 3′CS and 5′CS with internal variable region encoding a variety of antimicrobial resistance genes including aminoglycosideadenyl transferase (*aadA*), and the dihydrofolatereductase *(dfrA*), conferring resistance against streptomycin/spectinomycin and trimethoprim, respectively [5]. Also, different studies have reported that class II and III integrons are barely being isolated from *K. pneumoniae*, harboring different resistance genes and are responsible for resistance against a wide variety of antibiotics such as beta-lactam, aminoglycoside and trimethoprim, as well as erythromycin [3-5].
Owing to the great impact of integron-positive isolates on resistance to antibiotic and patients' outcomes, the aim of this study was to investigate the antibiotic resistance profiles and prevalence of class I, II, and III integrons among clinical isolates of *K. pneumoniae*.

MATERIALS AND METHODS

Sample collection and identification
A total of 142 non-duplicate, consecutive clinical isolates of *K. pneumoniae* were collected from patients referred to Sistan and Balouchestan hospitals during March 2018 and March 2019. Standard conventional tests and species-specific PCR were used to identify *K. pneumoniae* [6]. The study was approved by ethical committee of Zabol University of Medical Sciences (IR.ZBMU.REC.1397.179).

Antimicrobial susceptibility testing
Antibiotic susceptibility was assessed using Kirby-Bauer's disk diffusion method and Clinical and Laboratory Standards Institute guidelines [7]. Used antibiotics (MSAT, Merseyside, UK) were as follows: ciprofloxacin (CIP, 5 µg), meropenem (MEM, 10 µg), imipenem (IMP, 10 µg), amikacin (AMK, 30 µg), ertapenem (ETP, 10 µg), cefoxitin (FOX, 30 µg), cefepime (CPM, 30 µg), streptomycin (S, 10 µg), ceftriaxone (CRO, 30 µg) and gentamicin (GM, 10 µg). *Pseudomonas aeruginosa* ATCC 27853 were used as the quality control.

Molecular detection of Integrons by polymerase chain reaction (PCR)
The isolates were cultured (24h) on MacConkey agar at 37°C. Then, genomic DNAs were extracted by boiling method according to previous instructions [8]. Oligonucleotide primers used in this study are shown in Table 1. All PCR reactions were performed using Ampliqon (Denmark) ready to use master mix. For each integron, PCR reaction mixtures in final volume of 20 µL, comprised 15 µL ready to use master mix, 3 µL extracted DNA and 1 µL forward primer (100 pmol) and 1 µL reverse primer (100 pmol) were used [9]. PCR program was applied as follows: initial denaturation at 94°C for 6 min, 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 1 min and a final extension at 72°C for 10 min. Finally, The PCR products were separated using agarose gel electrophoresis (1%) and visualized after staining with Sybr safe (Thermo Fisher Scientific Inc.).

Table 1 - Oligonucleotide primers used in this study.

Primer	*Nucleotide sequence (5′….3′)*	*PCR target*	*Expected size (bp)*	*Reference*
Inti1F	CCT CCC GCA CGA TGA TC	*intI1*	280	9
Inti1R	TCC ACG CAT CGT CAG GC			
Inti2F	TTA TTG CTG GGA TTA GGC	*intI2*	233	9
Inti2R	ACG GCT ACC CTC TGT TAT C			
Inti3F	AGT GGG TGG CGA ATG AGT G	*intI3*	600	9
Inti3R	TGT TCT TGT ATC GGC AGG TG			

Statistical analysis
Statistical analysis of results was carried out with SPSS software (SPSS Chicago, IL). The Chi-square test and Fisher's exact test were used for statistical analysis. A *P* value <0.05 was considered statistically significance.

RESULTS

Distribution of sources of collected samples was as follows: urine 69 (48.6%), sputum 53 (37.3%), blood 8 (5.6%), wound 5 (3.5%) and other clinical specimens (synovial fluid, abscess and eye discharge) 7 (4.9%). From these, fifty-seven samples (40.1%) were taken from female and 85 (59.9%) from male patients. Also, out of 142 isolated bacteria, 51 (35.9%) were from outpatients and 91 (64.1%) were from hospitalized patients. The isolates were mostly resistant to streptomycin (62 strains, 43.7 %) and ceftriaxone (42 strains, 29.6%), followed by ciprofloxacin (39 strains, 27.5%) and cefepime (37 strains, 26.1%) (Table 2). Twenty-six (18.3%) isolates were found to be MDR, showing at least resistance against three antibiotics of different classes such as aminoglycoside, beta-lactams and quinolones. Class I and II integrons were detected in 65 (45.8%) and 1 (0.7%) isolates, respectively. Also, one isolate had class I and II integron,

Table 2 - Antibiotic resistance profiles of isolated *K. pneumoniae*.

Antibiotic	*Susceptible (n, %)*	*Intermediate (n, %)*	*Resistant (n, %)*
Meropenem	127 (89.4)	2 (1.4)	13 (9.2)
Imipenem	127 (89.4)	2 (1.4)	13 (9.2)
Ertapenem	127 (89.4)	2 (1.4)	13 (9.2)
Streptomycin	74 (52.1)	6 (4.2)	62 (43.7)
Amikacin	121 (85.2)	3 (2.1)	18 (12.7)
Gentamicin	98 (69)	0 (0)	44 (31)
Cefoxitin	114 (80.3)	1 (0.7)	27 (19)
Cefepime	102 (71.8)	3 (2.1)	37 (26.1)
Ceftriaxone	97 (63.8)	3 (2.1)	42 (29.6)
Ciprofloxacin	85 (59.9)	18 (12.7)	39 (27.5)

Table 3 - Association between antibiotic resistance and integron carriage in clinical isolates of *K. pneumoniae*.

Antibiotic	*Integron-positive isolates (n = 65)*			*Integron-negative isolates (n = 77)*			*Total isolates (n = 142)*			*P value*
	S (%)	*I (%)*	*R (%)*	*S (%)*	*I (%)*	*R (%)*	*S (%)*	*I (%)*	*R (%)*	
Meropenem	50 (76.9)	2 (3.1)	13 (20)	77 (100)	0 (0)	0 (0)	127 (89.4)	2 (1.4)	13 (9.2)	*0.000*
Imipenem	50 (76.9)	2 (3.1)	13 (20)	77 (100)	0 (0)	0 (0)	127 (89.4)	2 (1.4)	13 (9.2)	*0.000*
Ertapenem	50 (76.9)	2 (3.1)	13 (20)	77 (100)	0 (0)	0 (0)	127 (89.4)	2 (1.4)	13 (9.2)	*0.000*
Streptomycin	18 (27.7)	5 (7.7)	42 (64.6)	56 (72.7)	1 (1.3)	20 (26)	74 (52.1)	6 (4.2)	62 (43.7)	*0.000*
Amikacin	49 (75.4)	1 (1.5)	15 (23.1)	72 (93.5)	2 (2.6)	3 (3.9)	121 (85.2)	3 (2.1)	18 (12.7)	*0.003*
Gentamicin	32 (49.2)	0 (0)	33 (50.8)	66 (85.7)	0 (0)	11 (14.3)	98 (69)	0 (0)	44 (31)	*0.000*
Cefoxitin	44(67.7)	0 (0)	21 (32.3)	70 (90.9)	1 (1.3)	6 (7.8)	114 (80.3)	1 (0.7)	27 (19)	*0.001*
Cefepime	36 (55.4)	0 (0)	29 (44.6)	66 (85.7)	3 (3.9)	8 (10.4)	102 (71.8)	3 (2.1)	37 (26.1)	*0.000*
Ceftriaxone	36 (55.4)	0 (0)	29 (44.6)	61 (72.9)	3 (3.9)	13 (16.9)	97 (63.8)	3 (2.1)	42 (29.6)	*0.001*
Ciprofloxacin	23 (35.4)	10 (15.4)	32 (49.2)	62 (80.5)	8 (10.4)	7 (9.1)	85 (59.9)	18 (12.7)	39 (27.5)	*0.000*

S, susceptible; I, intermediate; R, resistant. Significant values are in bold.

simultaneously. Class III integron was not found. As shown in Table 3, the association between integron carriage and antibiotic resistance was most significant for cefepime, meropenem, imipenem, ciprofloxacin, gentamicin and streptomycin ($P \leq 0.001$). Moreover, the comparison of MDR phenotype in the integron-positive and -negative isolates revealed that integron-positive isolates had much higher rate of MDR (88.5%) compared with integron-negative (11.5%) isolates ($P<0.001$).

DISCUSSION

Infections caused by *K. pneumoniae* strains pose serious threat to human health worldwide owing to the great ability of this microorganism to produce different virulence factors and to develop antibiotic resistance. Therefore, we investigated the antibiotic resistance patterns and prevalence of class I, II and III integrons among clinical isolates of *K. pneumoniae*. In this study, *K. pneumoniae* showed varying levels of resistance against the tested antibiotics revealing that carbapenems are the most effective antibiotics against *K. pneumoniae*, with 89.4% of all isolates being susceptible. A comprehensive meta-analysis study conducted in Iran showed that the prevalence of carbapenem resistant *K. pneumoniae* (CR *K. pneumoniae*) was about 11%, ranging from less than 1% to 58% [10]. Our findings on the prevalence of CR *K. pneumoniae* were higher than that of some European countries such as Estonia (0%), Sweden (0%), Finland (0%), Germany (0.1%), Norway (0.1%) and Netherlands (0.1%), whereas the prevalence of CR *K. pneumoniae* in Italy and Greece was reported to be higher than that of our results, with 33.5% and 61.5% resistance rate, respectively [11].

These contradictory findings may be related to the widespread use of antibiotics, variable sample sizes, poor infection control policies, differences in geographical locations and the presence of some risk factors such as previous hospitalization and long-term staying at ICU [12, 13].

The findings revealed that isolates were mostly resistant against streptomycin, gentamicin and ceftriaxone, with 43%, 31% and 29% of isolates being resistant, respectively. The prevalence of antimicrobial resistance isolates is not same in different regions of Iran. For instance, Ranjbar et al, in their study which was conducted in Isfahan province, reported that 53%, 60%, and 80% of the investigated *K. pneumoniae* isolates were resistant to gentamicin, amikacin and ciprofloxacin, respectively [14]. Moreover, it has been reported that the percentage of resistance to different antibiotics in different provinces of Iran is much higher than that of most European countries. For example, resistance to gentamicin varied from 6.2% in Rasht province to 69.8% in Tabriz province. Also, resistance to other antibiotics such as ciprofloxacin varied from 20% in Zahedan province to 53.2% in Isfahan province [10].

Different antibiotic resistance rates probably resulted from indiscriminate use of antibiotics. Also, it has been reported that previous hospitalization at ICU, presence of central line device and exposure to antibiotics are associated with higher rates of antibiotic resistance [8, 10].

Several different mechanisms have been identified that can confer resistance to different antibiotics belonging to different classes. The production of extended-spectrum beta-lactamase (ESBL)-enzymes, efflux pump overexpression, target modifying enzymes and mutation are the most important mechanisms that have been identified in *K. pneumoniae* [15].

In the next step of this study, the prevalence of class I (*intl1*), class II (*intl2*) and class III (*intl3*) integrons were investigated. PCR results showed that 45.8% (n=65) and 0.7% (n=1) of isolates had *intl1* and *intl2* genes, respectively; but *intl3* was not found in the studied isolates.

The findings of different studies demonstrated that the *intl1* is the most predominant integrons among *K. pneumoniae* strains. For instance, Li et al., in a study conducted in China, reported that 51.1% (90/176) of investigated *K. pneumoniae* had *intl1* genes [16]. Likewise, two independent studies conducted in Yazd province and Kashan province (Iran), reported that the prevalence of *intl1* was 91% and 100%, respectively [17, 18].

The spread of integron-positive isolates in hospital may lead to the dissemination of multi-drug resistant isolates, because these mobile genetic elements often contain multiple drug resistance genes that can be disseminated among bacteria through horizontal gene transfer [19, 20].

Also, our findings showed significant association between *intl1* and resistance to cefepime, meropenem, imipenem, ciprofloxacin, gentamicin and streptomycin ($P \leq 0.001$). It has been reported that there is a significant relationship between inte-

grons and resistance to antibiotics. A study conducted by Xu et al., reported results similar to ours revealing that the prevalence of resistance against different antibiotics including gentamicin, amikacin, streptomycin and ciprofloxacin in *intl1* positive isolates was significantly higher than that of integron negative isolates [19].
It is well documented that class I integrons play a significant role in the dissemination of antibiotic resistance in clinical bacterial isolates. In fact, the great ability of integrons in capturing multiple drug resistance genes has led to the emergence of class I integrons with different gene cassette arrays, encoding different resistance genes which may confer resistance against different drugs [20].

CONCLUSIONS

In order to impede the emergence of infections caused by antibiotic resistance bacteria and launch appropriate infection control policies, constant monitoring of antibiotic resistance profiles is of pivotal importance. Based on the results of this study, the prevalence of resistance to imipenem, meropenem and ertapenem was not at high level, however, resistance to streptomycin was high. Integrons appear to comprise one of the common features among *K. pneumoniae* isolates in our region, especially among MDR strains, and are associated with a high prevalence of antibiotic resistance. Accurate and rapid detection of integron harboring isolates is completely necessary, because other drug resistance genes are often located at these mobile genetic elements and may confer resistance against multiple drugs.

Conflict of interest
None

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Ethical approval
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